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021 Antigenic Analysis of Hematopoiesis: II. Expression of Human 022 Neutrophil Antigens on Normal and Leukemic Marrow Cells

023 Lewis C. Strauss, Keith M. Skubitz, J. Thomas August, and Curt I. Civin

024 The binding of five antineutrophil monoclonal autobodies, 025 AHN-1, -2, -3, -7, and -8, to normal and leukemic bone 026 marrow cells was studied. AHN-7 bound to many granulo- 027 cytic precursors, particularly myelocytes, and both lym- 028 phoid and blast cells in normal marrow, and to most but not 029 all granulocyte-macrophage progenitors (CFC-GM). AHN-8 030 bound only to late (band and segmented) neutrophilic cells 031 and not to CFC-GM. AHN-1, -2, -3 bound to morphologically 032 identifiable neutrophil precursors, but not to (day-14) CFC- 033 GM. Approximately half of nonlymphoid leukemia speci- 034 mens were positive with AHN-1 or AHN-7; by contrast, 035 lymphoid leukemia specimens were rarely positive. AHN-8 036 was rarely found on leukemic cells. These antineutrophil 037 antibodies appear to detect distinct granulopoietic subsets 038 and may be useful in the analysis of hematologic differen- 039 tiation and in the subclassification of leukemias.

040 HYBRIDOMA-DERIVED monoclonal antibodies
041 (MoAb) specifically reactive with lymphocyte
042 cell surface molecules have been of great value in the
043 analysis of lymphocyte differentiation and lymphoid
044 neoplasia.[1] MoAb reactive with human neutrophils
045 have been developed[2] and are potentially important
046 tools for the study of granulocyte function,[3] leukemic
047 cell origins,[4] and granulopoiesis.[5] Antibodies against
048 the My-1 human granulocyte antigen react with mor-
049 phologically identifiable neutrophil precursors, but not
050 with colony-forming cells of the granulocyte-monocyte
051 lineage (CFC-GM).[6] We have studied five additional
052 antineutrophil monoclonal antibodies for reactivity
053 with human leukemic and normal marrow cells,
054 including CFC-GM. The AHN-7 MoAb reacts with a
055 neutrophil surface protein of ~~45,000/65,000~~ apparent
056 molecular weight and binds to peripheral blood baso-
057 phils, ~~eosinophils~~, and monocytes, as well as neutro-
058 phils[7] (and unpublished data). AHN-8 recognizes a
059 glycolipid antigen and reacts solely with mature neu-
060 trophils in peripheral blood.[7] AHN-1, -2, and -3 bind
061 to a carbohydrate sequence found on several mem-
062 brane glycolipids and ~~six~~ proteins of neutrophils.[7,8]

063 MATERIALS AND METHODS

Note: we would prefer MoAb as an abbreviation for monoclonal antibody (throughout the paper).

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Neutrophil Antigens on Marrow Cells

(28,000 - 65,000) as well as glycolipids,

URGENT

PLEASE CHECK AND RETURN YOUR GALLEYS WITHIN 48 HOURS. THE ISSUE IS RUNNING LATE.

THANK YOU.

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Murine monoclonal antibodies AHN-1, -2, -3, -7, -8 were prepared as previously described.[7,8] AHN-1, -2, and -3 are IgM(κ) MoAb; AHN-7 and -8 are IgG1(κ) MoAb. Neat spent hybridoma culture supernatant was used as the antibody source for AHN-1, -3, -7, -8; diluted ascites fluid (1:50) was used for AHN-2. Negative controls were the IgM myeloma protein, TEPC 183, used as diluted ascites fluid (1:50), and MOPC 21, a IgG1 myeloma protein, used as diluted ascites (1:50) or neat spent supernatant of the P3X63.AG8 cell line (American Type Culture Collection, Rockville, MD). These isotype-matched myeloma proteins react with no known antigens and were utilized in parallel with AHN MoAbs to control for the possible binding of MoAb due to the Fc rather than the Fab region. The IgG2b MoAb, 28/43/6, which binds to lymphocytes from all donors tested,[6] was used as a positive control. All antibodies were used in greater than eightfold excess.

Blood cells and bone marrow cells were prepared, and indirect immunofluorescence assays were performed as previously described.[6] Background fluorescence obtained with negative control antibodies was <5% and was subtracted from that obtained with AHN MoAb. Low-density nonadherent human bone marrow leukocytes [5 × 10^6/ml in RPMI 1640 (Flow, Rockville, MD) containing 0.2% bovine serum albumin (BSA; Sigma, St. Louis, MO)] were routinely incubated with an equal volume of sterile, centrifuged (15,600 g, 15 min, 4°C) MoAb for 20 min (22°C). The cells were washed twice, then either (A) "panned" on a goat anti-mouse immunoglobulin-coated Petri dish, using the previously described immune adherence "panning" technique,[6,9] or (B) resuspended in RPMI 1640 containing 0.2% BSA, DNAse I (250 Kunitz U/ml; Sigma, St. Louis, MO) and rabbit complement (Cedarlane "Low-Tox H," Accurate Chemical Corp., Westbury, NY) at 1:8 dilution, as described.[6] Cells recovered from these procedures were counted (viable cell count by trypan blue dye exclusion), examined for morphology, and placed in semisolid agar tissue culture medium (containing 5% v/v human placenta conditioned medium) for growth and enumeration of CFC-GM.[10]

RESULTS

Normal human marrow leukocytes were examined for reactivity with the AHN-7 antibody. Low-density nonadherent marrow leukocytes were separated by immune adherence ("panning") into antigen-positive (bound) and antigen-negative (unbound) populations. Aliquots of the panned cell populations were incubated (again) with AHN-7 antibody, then analyzed by indirect immunofluorescence for expression of AHN-7 antigen: >90% of the cells in the bound fraction were AHN-7-positive, compared with 35% in the unseparated population; marked variation in fluorescence staining intensity was observed. Approximately 10% of the cells in the unbound fraction were (weakly) AHN-7-positive.

Fractions obtained after treatment with excess AHN-7 and panning were examined morphologically and assayed for CFC-GM. The AHN-7-positive (bound) marrow cell fraction (Table 1A) contained morphologically identifiable eosinophil, basophil, and neutrophil precursor cells, including myeloblasts, and was particularly rich in neutrophilic myelocytes (32% and 31% in 2 experiments). A large number of lymphoid cells and blast cells of several lineages were also seen in the bound fraction. Thus, most or all myelocytes and subsets of these other marrow cell types express the antigen identified by AHN-7. The AHN-7-negative (unbound) population was depleted of CFC-GM (Table 2A): only 21% (21%–38% in 3 experiments) of control CFC-GM remained. In contrast, with the positive control MoAb 28/43/6, over

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136 90% of the marrow leukocytes and all of the CFC-GM
137 were bound.[4] The AHN-7-positive (bound) population
138 contained 43% of the starting CFC-GM (19%–43% in
139 4 experiments), suggesting that most, but not all,
140 CFC-GM in normal marrow express the antigen
141 detected by AHN-7. This was not due to nonspecific
142 binding of CFC-GM, as no CFC-GM were ever
143 detected in the small cell populations bound after
144 negative control antibody treatment (n = 8 experi-
145 ments).

146 Marrow leukocytes isolated by treatment with
147 AHN-8 MoAb and panning were predominantly
148 (>80% in all 6 experiments) late neutrophilic forms:
149 22% metamyelocytes, 32% band, and 40% segmented
150 polymorphonuclear leukocytes in the experiment
151 shown (Table 1B). In 6 replicate panning experiments
152 with AHN-8, average cell recovery in the antigen-
153 positive population was 12% (range 7%–14%) of the
154 starting cells. As expected, when these morphologi-
155 cally well differentiated AHN-8-positive cells were
156 cultured (Table 2B), CFC-GM were absent, and CFC-
157 GM were recovered quantitatively in the antigen-
158 negative fraction (recovery 86% ± 18%, n = 6 experi-
159 ments). The results indicate that expression of the
160 AHN-8 antigen by hematopoietic cells is confined to
161 the most morphologically mature cells in the neutro-
162 philic series.

163 The bound population of marrow leukocytes after
164 treatment with MoAb AHN-1, -2, or -3, contained
165 approximately 75% morphologically identifiable neu-
166 trophil precursors, including progranulocytes, plus
167 rare myeloid blasts. The unbound population con-
168 tained predominantly erythroid and lymphoid cells, as
169 well as small numbers of eosinophils, basophils, and
170 megakaryocytes (Table 1C; results for AHN-2 and
171 AHN-3 were essentially identical to those for AHN-1
172 and are omitted from Table 1, ~~C and D~~). When the
173 panned populations were cultured, CFC-GM were
174 recovered quantitatively in the antigen-negative frac-
175 tions and were absent from antigen-positive fractions
176 (Table 2C).

177 The IgM MoAb AHN-1, -2, and -3 were also tested
178 for cellular reactivity by complement-mediated cytoly-
179 sis. These MoAb were strongly cytotoxic to HL-60
180 target cells, as measured either by trypan blue dye
181 exclusion[8] or by colony-formation assay (data not
182 shown). Viable marrow cells (isolated by density-
183 gradient centrifugation) remaining after cytolysis with
184 AHN-1, -2, or -3 antibody plus complement were
185 greatly enriched for erythroid, lymphoid, and blast
186 cells (Table 1D); essentially all morphologically identi-
187 fied neutrophil precursors were removed. However, no
188 reduction of CFC-GM numbers was observed, either
189 in individual experiments or in pooled data from
190 multiple experiments (Table 3A). AHN-2 was tested
191 as diluted (1:50) ascites fluid; the slight observed effect
192 of AHN-2 on CFC-GM numbers may be due to
193 unknown substances in the ascites fluid. In contrast,
194 CFC-GM were almost completely eliminated by posi-
195 tive control MoAb 28/43/6 plus complement under
196 these conditions. Marrow cells surviving initial treat-

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200 ment with AHN antibody plus complement were
201 treated a second time with fresh antibody plus comple-
202 ment; no incremental effect on CFC-GM number was
203 observed (Table 3B), confirming that initial conditions
204 included excess antibody and complement. Antibody
205 excess was demonstrated directly by preincubation of
206 MoAb with marrow leukocytes prior to use in anti-
207 HL-60 cell cytolysis experiments: marrow-preab-
208 sorbed antibody was as strongy cytolytic for HL-60
209 cells as unabsorbed antibody (data not shown). These
210 results suggest that the antigen(s) identified by AHN-
211 1, -2, and -3 are expressed by morphologically identifi-
212 able neutrophilic precursors, but not by cells of other
213 lineages nor by day-14 CFC-GM.
214 Leukemic blast cell specimens were analyzed by
215 indirect immunofluorescence (as previously de-
216 scribed[6]) for expression of these AHN MoAb. Only
217 specimens containing ≥80% leukemic cells were ana-
218 lyzed. A finding of ≥20% fluorescent cells (above
219 background with isotype-matched control MoAb)
220 indicated expression of an antigen by the leukemic
221 cells and was defined as a positive specimen. MoAb
222 AHN-1 and AHN-7 reacted with approximately half
223 (55% and 49%, respectively) of the specimens from
224 patients with morphologically defined acute nonlym-
225 phoblastic leukemia (Table 4). A single infant with
226 CALLa-negative acute lymphoid leukemia (ALL) was
227 positive for AHN-1. Binding of certain antineutrophil
228 MoAb to rare ALL blast cell specimens has been
229 previously noted.[11] AHN-8 reacted with only 1 of 34
230 ANLL and no ALL patients tested; the sole AHN-
231 8-positive patient was only marginally positive (22%
232 fluorescent cells) and was AHN-8-negative at relapse.

233 ## DISCUSSION

234 It appears that AHN-7, which identifies an antigen
235 on neutrophils, ~~eosinophils~~, basophils, and monocytes
236 in peripheral blood, identifies the precursors of these
237 cells and also a subset of mononuclear cells, including
238 blast cells in normal marrow. Cells at the myelocyte
239 stage were most uniformly positive for AHN-7 in
240 panning experiments, suggesting that antigen expres-
241 sion is maximal at this point in development. The
242 observation that only 54% of the CFC-GM "missing"
243 from the unbound fraction was detected in the bound
244 fraction can be attributed, at least in part, to loss of
245 viable cells. The recovery of bound cells required
246 vigorous pipetting; some mechanically induced reduc-
247 tion of colony-forming efficiency might therefore be
248 expected. In the experiment shown, 2 ml of neat
249 AHN-7 supernatant was used, fourfold excess volume
250 over that used in usual experiments, to assure MoAb
251 excess. Partition of antigen-positive from antigen-
252 negative cells was demonstrated by immunofluores-
253 cence assay, but some weakly positive cells were still
254 unbound. The purity of the antigen-negative fraction
255 may depend on the physical method used to collect
256 unbound cells. Conversely, the purity of the bound
257 fraction may depend on the presence of Fc-receptor-
258 bearing cells in the suspension. As no CFC-GM were
259 unbound after treatment with positive control MoAb

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28/43/6, however, and no nonspecific binding of CFC-GM was observed, these experiments suggest that CFC-GM in normal marrow are at least quantitatively heterogeneous for expression of the antigen identified by AHN-7. An alternative hypothesis, which we consider less likely, is that a helper cell population[12] was partitioned from the CFC-GM by the procedure. Furthermore, we suggest that the AHN-7-positive population does not correspond to a single morphologically defined cell category, but may relate to the proliferative state as has been shown for the My-7 antigen.[13] The AHN-7 antigen may prove to be important in the further analysis of hematopoiesis.

The antigen identified by AHN-8 is expressed strongly only very late in neutrophilic maturation and might be important in surface-dependent mature neutrophil function. The MoAb AHN-1, -2, and -3 identify a cell population very similar to that defined by the glycolipid[14] My-1 differentiation antigen of human neutrophils. Normal cells of lineages other than neutrophilic were not identified by AHN-1, -2, or -3, in contrast to AHN-7. Immature neutrophils were AHN-1-positive (but AHN-8-negative), but CFC-GM were AHN-1-negative. The results of cell separation using AHN-1, -2, -3 and panning were confirmed using complement-mediated cytotoxicity. The removal of antibody-positive cells was more complete using complement, but the results of CFC-GM assays were identical, showing high CFC-GM recovery in both antibody-negative populations. Another laboratory[7] has observed that CFC-GM express AHN-1, -2, -3 antigens. Possible explanations for these contrasting results are that the other studies used a different source of colony-stimulating factor (leukocyte conditioned media), a different preparation of MoAb (ascites), and a different duration of culture for CFC-GM (8 days).

In the analysis of ANLL blast cell specimens with antimyeloid MoAb, normal granulopoietic cells expressing the detected antigens might contaminate the leukemic cells. False positive results arising in this way were excluded by the requirement that ≥20% (above background) of cells be fluorescent in a sample containing ≥80% leukemic cells. Thus, the percentages of positive specimens shown (Table 4) are minimum estimates. As many ANLL specimens reacted with AHN-1 and AHN-7, these antibodies might, when positive, be helpful in the distinction of ANLL from ALL. It is intriguing that ANLL blast cells rarely (if ever) bind AHN-8. Conceivably, AHN-8-positive cells may not be susceptible to leukemic transformation, or, once transformed, the leukemic cells may obligately lose this antigen. Alternatively, nonlymphocytic leukemic cells may be unable to differentiate to the stage of AHN-8 antigen expression.[15] Whatever the mechanism, the rarity of AHN-8-positive ANLL blast cells is analogous to the expression of surface antigens (e.g., T3, surface immunoglobulin) of normal mature lymphoid cells only on rare ALL blast cells.[1]

We have shown that the MoAb AHN-1, -2, and -3 detect a lineage- and stage-specific neutrophil differ-

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327 entiation antigen very similar to the My-1 antigen[4] not
328 present on day-14 CFC-GM. The AHN-7 MoAb
329 detects an antigen expressed by maturing granulocytic
330 cells, by many lymphoid and blast cells, and by many,
331 but not all, CFC-GM. The AHN-8 MoAb detects an
332 antigen expressed very late in neutrophil differentia-
333 tion, first on metamyelocytes. As these AHN antibod-
334 ies define distinct, but overlapping, sets of granulo-
335 poietic cells, they are of potential use in the study of the
336 mechanisms of normal cellular differentiation and the
337 aberrant differentiation processes in leukemia.

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Table 1. Differential Counts of AHN-Treated Marrow Cell Specimens*

008		Blast (%)	Progranulocyte (%)	Myelocyte (%)	Meta + Band + Poly† (%)	Lymphoid (%)	Erythroid (%)	Monocyte (%)	Eosinophil (%)	Basophil‡ (%)
016	(A) AHN-7 "panned"									
026	Exp. 1									
036	MOPC 21—unbound	11	6	18	33	21	8	8	0.5	0.5
046	AHN-7—unbound	7	6	3	36	26	15	4	0	0
056	AHN-7—Bound	10	9	32	18	29	0	2	1	1
066	Exp. 2									
076	MOPC 21—unbound	13	10	18	12	19	26	2	0.5	0.5
086	AHN-7—unbound	13	10	6	6	17	44	4	0	0
096	AHN-7—bound	16	4	31	10	36	1	1	0.5	1
106	(B) AHN-8 "panned"									
116	MOPC 21—unbound	6	6	18	51	18	1	0	0	0
126	AHN-8—bound	0	0	1	94	5	0	0	0	0
136	(C) AHN-1 "panned" §									
146	TEPC 183—unbound	9	13	7	16	26	21	2	0.5	0.5
156	AHN-1—unbound	13	3	3	4	37	35	4	0.5	0.5
166	AHN-1—bound	2	20	12	42	19	3	1	0	0
176	(D) AHN-1 cytolysed§									
186	TEPC 183 + complement	8	14	18	14	43	8	1	0	0
196	AHN-1 + complement	9	0	0	0	78	13	2	0	0

208 *Percent of cells tested on 500–2,000 cell differential counts (rounded) of Wright–Giemsa-stained smears from a representative experiment.
207 †Metamyelocytes, band forms, and polymorphonuclear leukocytes.
208 ‡Eosinophil and basophil were defined by characteristic secondary granulation; thus, myelocytes and more mature cells in each lineage were counted as eosinophils and basophils, respectively.
209 §Results obtained after treatment with AHN-2 or AHN-3 were essentially identical to AHN-1.

210 **Table 2. Separation By "Panning" of AHN Antibody-Treated**
211 **Marrow Cells: CFC-GM in Positive and Negative Fractions**

212 213		Viable Cell Recovery*	CFC-GM/10^5 Cells†	CFC-GM Recovered‡
217	(A) MOPC 21	88	23 (5)	2,000
221	(sup§)—unbound			
225	MOPC 21 (sup)—bound	<1	ND	ND
229	AHN-7—unbound	52	8 (3)	412
233	AHN-7—bound	34	25 (3)	860
237	(B) MOPC 21	84	46 (3)	3,820
241	(asc§)—unbound			
245	MOPC 21 (asc)—bound	12	0 (0)	0
249	AHN-8—unbound	82	48 (2)	3,940
253	AHN-8—bound	14	1 (0)	20
257	(C) TEPC 183—unbound	98	77 (10)	8,320
261	TEPC 183—bound	3	0 (0)	0
265	AHN-1—unbound	62	136 (2)	9,280
269	AHN-1—bound	21	<1 (0)	10
273	AHN-2—unbound	47	172 (8)	8,920
277	AHN-2—bound	18	2 (1)	30
281	AHN-3—unbound	62	129 (10)	8,800
285	AHN-3—bound	16	2 (1)	30

289 *Values represent 100% × (Viable cell number in fraction ÷ Initial cell
290 number) after treatment with antibody and panning procedure.
291 †Mean (standard deviation) of triplicate determinations, rounded to
292 integer).
293 ‡Product of CFC-GM/10^5 cells × number viable cells in fraction.
294 §MOPC 21 (sup), neat supernatant of P3X63.AG8; MOPC 21 (asc),
295 diluted (1:50) ascites fluid.
296 ND, not done.

300 **Table 3**

301 (A) Treatment of Normal Bone Marrow Cells With AHN-1, 2, 3
302 Antibody Plus Complement: Effect on CFC-GM

303 Antibody	Viable Cell Recovery* (%)	Colony Count Single Exp.†	Colony Count Pooled Exp.‡
310 TEPC 183	96 (8)	62 (4)	(100) (0)
314 AHN-1	41 (4)	61 (9)	93 (8)
318 AHN-2§	50 (14)	40 (4)	79 (9)
322 AHN-3	72 (4)	60 (5)	94 (7)
326 28/43/6			
330 (positive control)	15 (3)	0 (0)	1 (1)

334 (B) CFC-GM in Residual Marrow Cells After Double-Treatment
335 With Antibody Plus Complement

		Colony Count	
337 Antibody (1)	Antibody (2)	Mean (SD)†	Percent of Control
342 None	None	93 (6)	82
346 TEPC 183	TEPC 183	114 (5)	[100]
350 AHN-1	AHN-1	143 (21)	125
354 AHN-2§	AHN-2§	85 (9)	75
358 AHN-3	AHN-3	101 (5)	89
362 TEPC 183	28/43/6	0 (0)	0

366 *Values represent number of 100% × (Viable cells recovered ÷ Initial
367 cell number). Mean (standard deviation) for four experiments is shown.
368 †Representative experiment with marrow from a normal donor. Mean
369 (standard deviation) of triplicate cultures of 10^5 cells is shown.
370 ‡Values represent mean (SEM) of colony counts from different
371 experiments (n = 11) expressed as percent of negative control (TEPC
372 183) in the same experiment.
373 §Antibody used as diluted (1:50) ascites fluid.

374 **Table 4. Binding of AHN Antibodies to Leukemic Cell Specimens**

376 Disease*	Percent Positive Specimens† AHN-1	AHN-7	AHN-8
380 Acute nonlymphoblastic leukemia	55% (23/42)	49% (19/39)	3% (1/34)
388 Acute lymphocytic leukemia			
392 CALLa-positive	0% (0/16)	0% (0/9)	0% (0/8)
400 HLA-DR-positive/CALLa-negative	33% (1/3)	0% (0/3)	0% (0/2)
408 T cell	0% (0/3)	0% (0/2)	0% (0/2)
416 Chronic myeloid leukemia			
420 Chronic phase	100% (1/1)	0% (0/1)	0% (0/1)
428 Blast crisis (myeloid)	100% (1/1)	50% (1/2)	0% (0/1)

436 *Acute nonlymphoblastic leukemia was defined morphologically;
437 acute lymphoid leukemia was defined morphologically and by expression
438 of CALLa, T and B lymphoid and HLA-DR surface markers (indirect
439 immunofluorescence). T cell: Leu-1 or T11 positivity was used to define T
440 lymphoid leukemia. Chronic myeloid leukemia was defined clinically and
441 by morphology.
442 †Percent positive specimens (number positive/number tested),
443 defined as ≥20% fluorescent cells above isotype-matched control
444 background fluorescence.